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Novel Nuclear Microsatellite Markers Reveal Genetic Diversity and Structure of *Veronicastrum sibiricum* var. *zuccarinii* (Plantaginaceae) in the Aso Region, Kyushu, Japan

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Veronicastrum sibiricum var. *zuccarinii* (Plantaginaceae) is an endangered perennial herb native to semi-natural grasslands in southern Korea and Kyushu, Japan. To elucidate the genetic status of *V. sibiricum* var. *zuccarinii* in the Aso region of Kyushu, in which relatively large populations still remain, we analyzed seven populations using five novel microsatellite markers. Each population of *V. sibiricum* var. *zuccarinii* has a maintained moderate level of genetic diversity (mean H_E = 0.596, mean N_A = 5.6, and mean AR = 4.7). Pairwise F_{ST} indicated significant genetic differentiation between all population pairs (0.065−0.309). The semi-natural grasslands in the Aso region have decreased in area over the past decades. Thus, habitat fragmentation may be causing genetic differentiation among populations of *V. sibiricum* var. *zuccarinii*. Although the populations of this variety have maintained a moderate degree of genetic diversity, the dynamics of gene flow among them should be continuously monitored.

Key words: conservation genetics, endangered species, grassland, habitat fragmentation, microsatellite, vegetative reproduction, *Veronicastrum sibiricum* var. *zuccarinii*

Anthropogenic disturbance is threatening the survival of many organisms globally (Pimm *et al*. 1995), with many species having gone extinct in the past decades (Lawton & May 1995, Regan *et al*. 2001, Smith *et al*. 1993). Even extant species are facing a reduction in population size (Groombridge 1992). It is estimated that without aggressive conservation measures, about two-thirds of all vascular plants may disappear by the end of the 21st century (Pitman & Jørgensen 2002). Approximately 7,000 vascular plant taxa are recognized in the Japanese flora, one-quarter of which is threatened by extinction (Ministry of the Environment of Japan 2007, 2012, 2015). Conservation efforts are therefore urgently needed for these threatened plant species.

Along with habitat destruction and commercial exploitation, stochastic processes, known as the "extinction vortex," have contributed to the destabilizing effects of species survival (Gilpin & Soulé 1986). Some of these stochastic factors are genetic, as the decline of genetic variation within populations can cause inbreeding depression and increase the risk of extinction (Frankham 2005). In addition, genetic drift may reduce the genetic variation of isolated populations and result in strong genetic differentiation among populations (Lienert 2004). In small or repeatedly bottlenecked populations, slightly deleterious mutations are expected to accumulate because of genetic drift, resulting in mutational meltdown and a rapid decline toward extinction (Lande 1995,

TABLE 1. The materials and their sources analysed for microsatellite variation of *Veronicastrum sibiricum* var. *zuccarinii* in Kyushu district, Japan.

Population	Locality*	Altitude (m)	No. of samples	Estimated population size
NA1	Namino, Aso, Kumamoto	760	35	~100
NA2	Namino, Aso, Kumamoto	780	35	~100
NA3	Namino, Aso, Kumamoto	770	16**	~100
NA4	Namino, Aso, Kumamoto	770	33	~100
TA1	Takamori, Kumamoto	870	35	~100
TA2	Takamori, Kumamoto	790	76	~500
KO	Kokonoe, Oita	800	4	~10
Total			234	

*From a view point of conservation, the approximate information of the localities is shown.

**In NA3 population, many ramets grew densely relatively in a small area, therefore the collected number of samples were relatively small.

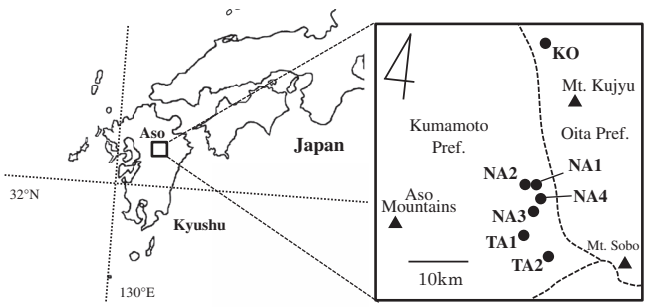

FIG. 1. Sampling locations of *Veronicastrum sibiricum* var. *zuccarinii* in the Aso region, Kyushu, Japan.

Lynch & Gabriel 1990). In threatened plant species, the reduction of genetic variation within populations may increase both the short-term risk of extinction, by reducing reproductive success (caused by pollen limitation and inbreeding depression) and the long-term risk of extinction by reducing the genetic capacity to respond to environmental changes (Frankham *et al*. 2002, Kery *et al*. 2000). Thus, conservation planning must take into account the genetic status of each population and individual (Neel & Cummings 2003).

Veronicastrum sibiricum (L.) Pennell var. *zuccarinii* (Koidz.) H. Hara (Plantaginaceae) is an endangered perennial herb of sunny meadows in the mountainous areas of southern Korea and northern Kyushu, Japan (Yamazaki 1993). Over the last few decades, changes in rural land use have greatly reduced and fragmented Japan's semi-natural grasslands, and many grassland species have become endangered (Tsukada *et al*. 2004), *V. sibiricum* var. *zuccarinii* being one of them. This variety has experienced a rapid population decline due to habitat loss resulting from abandonment of traditional grassland management and conifer plantation establishment, with only an estimated seven hundred individuals remaining (Environment Agency of Japan 2000). Based on the observed rapid decline in habitat

remnants and population numbers, var. *zuccarinii* (not the species as a whole) was categorized as "endangered" (EN) in the Japanese Red Data Book and List (Environment Agency of Japan 2000, Ministry of the Environment of Japan 2007). Recently, its status has been revised to "vulnerable" (VU; Ministry of the Environment of Japan 2012, 2015), because relatively large populations have been discovered recently in the Aso region. However, currently, population numbers are still limited.

Although *Veronicastrum sibiricum* var. *zuccarinii* is at risk of extinction, the population genetics of this variety have not been studied. In the present study, we developed and used microsatellite markers to identify the genetic structure and diversity of var. *zuccarinii*, including the fine-scale genetic structure within population. Highly variable microsatellite markers, which allow for accurate estimates of inter- and intra-populational differences, can detect populations with rare alleles that may be candidates for targeted conservation efforts, as well as populations with low diversity that potentially require management assistance (Frankham *et al*. 2002). We sampled extant populations from the Aso region. The data obtained will help inform the conservation and restoration of *V. sibiricum* var. *zuccarinii*.

Materials and Methods

Plant sampling

In July and August 2009–2011, we collected mature leaf tissues of *Veronicastrum sibiricum* var. *zuccarinii* from seven populations (NA1–NA4, TA1, TA2, and KO) in the Aso region of Kumamoto and Oita prefectures, Kyushu, Japan (Table 1, Fig. 1). The collections comprised almost all known populations in this region, including newly discovered populations (NA3 and NA4). Sampling was randomly performed throughout each population. A total of 234 individuals was collected. All samples were dried and preserved with silica gel. Population sizes were estimated for each population according to the number of flowering plants observed. Voucher specimens used in the present study were deposited in the herbarium of the Faculty of Science, Kumamoto University (KUMA).

For the TA2 population, we established an 18 × 16 m quadrat containing 444 individuals (the number of ramets). Of these, 66 ramets were randomly selected for collection and the location of each was mapped (Fig. 2). The samples were preserved as above and used to evaluate the fine-scale genetic structure (distribution of genets) of the population of *Veronicastrum sibiricum* var. *zuccarinii*.

Isolation and characterization of microsatellite markers

The development of microsatellite markers in the present study was based on improved techniques for isolating codominant compound microsatellite markers (Kaneko *et al*. 2007, Lian *et al*. 2006, Lian & Hogetsu 2002). A restriction DNA library was constructed by digesting DNA with *Ssp*I or *Eco*RV, a blunt-end restriction enzyme. The restriction fragments were then ligated using a DNA ligation kit (Takara Bio) with a specific blunt-end adaptor (consisting of the 48-mer 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' along with the 8-mer 5'-ACCAGCCC-NH_2-3', in which the 3'-end was capped with an amino residue). Fragments were amplified from the *Ssp*I DNA li-

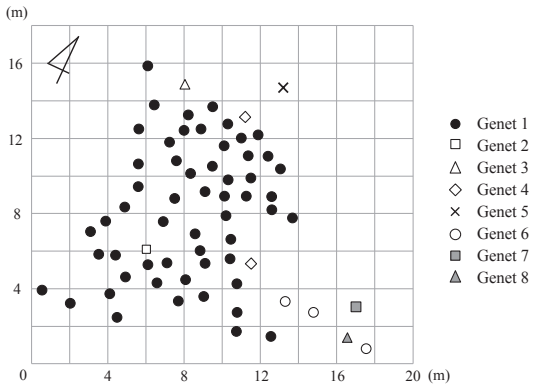

FIG. 2. Distribution of 66 ramets within the quadrat established in the TA2 population of *Veronicastrum sibiricum* var. *zuccarinii* (see, Fig. 1 and Table 1). Same character indicates plants with identical genotypes; we considered these plants to be same individuals or clones.

TABLE 2. Characteristics of the five compound microsatellite loci for *Veronicastrum sibiricum* var. *zuccarinii* and their variability.

Locus	Repeat motif	Primer sequence (5'-3')	T_a (ºC)	Size range (bp)	*A*	H_O	H_E	Accession No.
Vsz011	$(AC)_6(AG)_{15}$	ACACACACACACAGAGAGAGAG	57	80−152	26	0.604	0.770	AB926409
		TTTACCAGTCTTTATCCAACGA						
Vsz016	$(AC)_6(AG)_{11}$	ACACACACACACAGAGAGAGAG	57	160−190	7	0.577	0.514	AB926410
		CATATCTTTGCAGTAGGAAGCA						
Vsz025	$(AC)_6(AG)_{13}$	ACACACACACACAGAGAGAGAG	57	148−208	24	0.805	0.775	AB926411
		AGTTACAGATTTAGCGGCCTAC						
Vsz047	$(AC)_6(AG)_8$	ACACACACACACAGAGAGAGAG	57	199−207	5	0.479	0.407	AB926412
		GTCCTGGCTTTGAGTAACCA						
Vsz226	$(AG)_6(AC)_{10}$	AGAGAGAGAGAGACACACACAC	57	223−227	7	0.501	0.516	AB926413
		GAACAATGCTCTGTAACCACAT						
Average					13.2	0.593	0.596	

T_a, annealing temperature; *A*, the number of allele; H_O, observed heterozygosity; H_E, expected heterozygosity

brary using the compound microsatellite primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer (5'-CTATAGGGCACGCGTGGT-3'). The amplified fragments, ranging from 400 bp to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara Bio) and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were subsequently cloned using the QIAGEN PCR Cloning plus Kit (Qiagen) according to the manufacturer's instructions. Briefly, polymerase chain reaction (PCR) products were ligated into the pDrive vector and transformed into QIAGEN EZ competent cells. Transformants were identified by blue/white screening on LB agar plates containing ampicillin, X-gal, and IPTG. Cloned fragments containing $(AC)_6(AG)_n$ or $(TC)_6(AC)_n$ compound microsatellite sequences were amplified from the plasmid DNA of the positive clones, using M13 forward and reverse primers. Based on the sequences obtained, 13 candidate primers were designed using Primer3 version 0.4.0 (Rozen & Skaletsky 2000). Six of the candidate loci (*Vsz011*, *Vsz016*, *Vsz025*, *Vsz047*, *Vsz205*, and *Vsz226*) were amplified using PCR and showed polymorphic band patterns within and among the populations (Table 2). Next, we checked the efficiency of the primers using 48 plants from four populations (NA1–NA4). The linkage disequilibrium test using FSTAT version 2.9.3.2 (Goudet 1995) found significant genotypic disequilibrium between *Vsz025* and *Vsz205* ($P<0.05$); therefore, the *Vsz205* locus was excluded from the present analysis. Additionally, significant departure from Hardy–Weinberg equilibrium (HWE) after Bonferroni correction was detected in *Vsz011*, *Vsz025*, and *Vsz226* in the NA1 population, as well as that of *Vsz047* and *Vsz205* in the NA4 population. However, no locus showed significant departure from HWE in all populations. Thus, a total of five loci (excluding *Vsz205*) was used to assess the genetic diversity of *Veronicastrum sibiricum* var. *zuccarinii* (Table 2).

Microsatellite genotyping

Genomic DNA of all samples was extracted from leaves using a modified version of the CTAB method (Doyle & Doyle 1987). The five aforementioned microsatellite loci were amplified by PCR using a QIAGEN Multiplex PCR Kit (Qiagen) (Table 2). Each 5 µL PCR reaction mixture contained 0.5 µL template DNA (approx. 1–5 ng), 1.0 µM primers, and 2.5 µL Master mix. The PCR program included initial denaturation at

95°C for 15 min followed by 30 cycles of 30 s at 94°C, 1 min 30 s at 57°C, 1 min at 72°C; these were followed by a final extension at 60°C for 30 min. PCR was performed with the Thermal Cycler 2720 (Applied Biosystems). Sizes of the PCR products were estimated using the CEQ^{TM} 8000 Analysis System with a DNA Size Standard Kit-400 (both from Beckman Coulter).

Data analysis

We sampled and genotyped 234 plants from the seven populations (Table 1). In the following analyses, the KO population data were added only in the Bayesian clustering analysis (see below) because the sample size was small (four individuals). To estimate genetic diversity within each population, we calculated the mean number of alleles per locus (N_A), the expected and observed heterozygosities (H_E and H_O), the number of private alleles (*Pr*) present only in a single population, and the summed number of rare alleles with frequencies less than 5% among the total population (*RA*), using GenAlEx version 6.2 (Peakall & Smouse 2006). Also, allelic richness (*AR*; El Mousadik & Petit 1996) and inbreeding coefficients (F_{IS}) were calculated using FSTAT version 2.9.3.2. The latter parameter, F_{IS}, was tested with 600 randomizations and by applying Bonferroni correction, to determine whether its value differed significantly from zero.

Recent bottlenecks in the populations were evaluated using BOTTLENECK ver. 1.2.02 (Piry *et al*. 1999). We simulated equilibrium conditions (1,000 replications) assuming the infinite allele mutation model (IAM) and the two-phase model (TPM) with parameters recommended by Piry *et al*. (1999) (95% single step mutations, variance among multiple steps, 12). We used the Wilcoxon signed rank test to determine whether a significant excess of heterozygosity existed.

Pairwise F_{ST} (Weir & Cockerham 1984) measures the degree of genetic differentiation among populations and was computed using FSTAT version 2.9.3.2. This was tested with 300 permutations to determine whether it differed significantly from zero. To test whether genetic differentiation among populations followed an isolation-by-distance (IBD) pattern, a Mantel test (Mantel 1967) was conducted using GenAlEx version 6.2. In this test, the latitude and longitude data were used to determine geographic distance among populations, and the formula $F_{ST}/(1-F_{ST})$ was used to determine the genetic distance. Relationships among populations were inferred using a neighbor joining (NJ) tree based on the genetic distance among populations D_A (Nei *et al*. 1983), generated by Population 1.2.30 beta (Langella 2007). The robustness of each branch was evaluated by bootstrap probability from 1,000 resamplings of the genotype dataset. To infer the genetic structure of the populations, Bayesian clustering was implemented using STRUCTURE version 2.3.3 (Pritchard *et al*. 2000). This approach estimates the number of clusters (K) and assigns each individual to a cluster. The population structure was simulated with values of K = 1–9 under the LOCPRIOR model with admixture model and correlated allele frequencies model (*F*-model). The *F*-model assumes that all clusters diverged from a common ancestral population and may have experienced different degrees of genetic drift (Falush *et al*. 2003). In this model, the amount of genetic drift for each cluster is described as the *F*-value. The LOCPRIOR model modifies the prior distribution for each individual's population assignment and allows the proportion of individuals assigned to a particular cluster to vary by location (Hubisz *et al*. 2009). A series of 20 independent runs for each value of K ranging from 1 to 9 was performed with 1×10^6 MCMC iterations after 1×10^5 burn-in periods. To identify the appropriate value of K, we compared the mean value of the log probability of data (Ln $P(D)$) for each K, according to Pritchard *et al*. (2000).

Estimation of the fine-scale genetic structure of V. sibiricum *var.* zuccarinii

To estimate the fine-scale genetic structure (distribution of genets) of *Veronicastrum sibiricum* var. *zuccarinii*, we genotyped a part of individuals (66 ramets) collected from the 18×16 m quadrat established within the TA2 population using the five microsatellite loci and methods de-

TABLE 3. Genetic diversity measurements of each population of *Veronicastrum sibiricum* var. *zuccarinii*.

Population	*N*	N_G	N_A	*AR*	*RA*	*Pr*	H_O	H_E	F_{IS}	*P* value of bottleneck analysis	
										IAM	TPM
NA1	35	16	5.8	4.9	10	4	0.613	0.646	0.085	0.031	0.031
NA2	35	19	10.2	7.1	28	16	0.656	0.701	0.090	0.813	0.813
NA3	16	12	4.0	3.7	5	1	0.683	0.565	-0.168	0.156	1.000
NA4	33	10	4.2	4.1	8	2	0.489	0.563	0.185	0.156	0.625
TA1	35	13	4.4	4.1	8	1	0.600	0.549	-0.054	0.156	0.625
TA2	76	12	4.8	4.4	9	2	0.517	0.556	0.113	1.000	1.000
KO	4	2	–*	–*	–*	3	–*	–*	–*	–*	–*
Average**		13.7	5.6	4.7	10.0	4.3	0.593	0.596			

N, number of samples; N_G,number of genets; N_A, mean number of alleles per locus; *AR*, allelic richness; *RA*, summed number of rare alleles; *Pr*, summed number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; IAM, infinite allele model; TPM, two phase model.

*In the KO population, the values of genetic diversity within population were not caluculated because the sample size was small.

**The averages were the value excluding the samples of KO population.

scribed above. We used GenAlEx version 6.2 to calculate the probability of identity, which estimates the average probability that two unrelated individuals drawn from the same randomly mating population will have the same multilocus genotype. Because the probability of identity estimated by all five loci was very small (3.28×10^{-4}), plants with identical multilocus genotypes were regarded as clones or genets derived from vegetative reproduction. We investigated genet distribution in terms of location within the quadrat and individual genotype.

Observation of underground parts

To characterize the mode of vegetative reproduction, we observed the underground parts of *Veronicastrum sibiricum* var. *zuccarinii*. In a private garden at Takamori-machi, Kumamoto Pref., the variety was cultivated in the range of approximately 1 m × 1 m, and the number of the ramets with inflorescence was approximately 10. We carefully dug up the parts from the stock (approximately 30 cm × 40 cm). After the removal of loose soil and rinsing, the underground parts were stored in 70% ethanol.

Results

Marker loci polymorphisms

The number of alleles per locus ranged from 4 for *Vsz047* to 26 for *Vsz011*, with an average of 13.2 (Table 2); a total of 66 alleles were detected. Observed heterozygosity (H_O) ranged from 0.479 for *Vsz047* to 0.805 for *Vsz025*, with an average of 0.593. Expected heterozygosity (H_E) ranged from 0.407 for *Vsz047* to 0.775 for *Vsz025*, with an average of 0.596. Nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB926409−926413.

Genetic diversity within populations

We collected 16−76 individuals from each population excluding the KO population. However, the number of genets (N_G) that shared genotypes at all five microsatellite loci ranged from 10 to 19 (Table 3). The same genets were not detected among different populations. In each population, we excluded clones from subsequent calculations of genetic diversity (genet-level analyses). The mean number of alleles across loci (N_A) ranged from 4.0 for NA3 to 10.2 for NA2, and allelic richness (*AR*) ranged from 3.7 for NA3 to 7.1

TABLE 4. Pairwise F_{ST} (below diagonal) and geographical distance (km; above diagonal) among six populations of *Veronicastrum sibiricum* var. *zuccarinii*.

	NA1	NA2	NA3	NA4	TA1	TA2
NA1		1.0	3.7	1.6	7.1	12.1
NA2	0.089*		3.7	2.2	6.8	12.3
NA3	0.240*	0.138*		2.3	3.6	8.6
NA4	0.171*	0.137*	0.218*		5.9	10.5
TA1	0.065*	0.105*	0.284*	0.275*		7.0
TA2	0.068*	0.142*	0.309*	0.188*	0.152*	

*Significant differences from zero are indicated ($P < 0.05$).

for NA2; mean values were 5.6 and 4.7, respectively (Table 3). Forty-two alleles (64%) were rare alleles (*RA*) with frequencies less than 5%. Out of a total of 66 alleles, 26 were private alleles (*Pr*), which were observed only in a single population. The mean number of private alleles per locus ranged from 0.2 for NA3 and TA1 to 3.2 for NA2, with an average of 0.8. H_O ranged from 0.489 for NA4 to 0.683 for NA3 (mean, 0.593), and H_E ranged from 0.549 for TA1 to 0.701 for NA2 (mean, 0.596). F_{IS} ranged from -0.168 for NA3 to 0.185 for NA4, and no inbreeding coefficient significantly differed from zero. In the KO population, two genets were detected in four samples and three private alleles were detected.

BOTTLENECK (tested by Wilcoxon's signed rank test) analysis indicated recent population bottlenecks in the NA1 population under the IAM and TPM (Table 3). There was no excess of heterozygosity in any of the other five populations under either model.

Genetic differentiation and structure among populations

Although the geographical distance among populations was small (1.0–12.3 km), significant genetic differentiation was observed in *Veronicastrum sibiricum* var. *zuccarinii*. Pairwise F_{ST} ranged from 0.065 between the NA1 and TA1 populations to 0.309 between the NA3 and TA2 populations (Table 4). F_{ST} values between all population pairs were significantly greater than zero ($P < 0.05$).

The analysis of genetic structure consistently showed that genetic differentiation among populations did not correspond to geographical distribution. The Mantel test did not indicate a positive correlation between geographic distance and genetic distance among populations ($R^2 = 0.005$, $P = 0.465$) (Fig. 3). The neighbor-joining tree based on genetic distances among populations (D_A) showed that all branches had bootstrap probabilities of 50% or less (Fig. 4), indicating that the relationships among the populations were unclear.

In Bayesian clustering, the mean log probability of the data (Ln $P(D)$) over 20 runs for each cluster was highest at $K = 7$ (Fig. 5). The result of a single run at $K = 7$ is shown in Figure 6. In all populations, the cluster with the highest proportion of membership differed (Clusters 1–7). The *F*-value of each cluster ranged from 0 to 0.53, the highest value of which occurred in cluster 7.

Quadrat investigation in the TA2 population

Analysis of 66 ramets collected from a quadrat established in the TA2 population, based on multilocus genotypes of five microsatellite markers, revealed that these ramets consisted of 8 genets (Fig. 2). Among the ramets, 56 were members of Genet 1, three of Genet 6, and two of Genet 4. The remaining ramets were members of Genets 2, 3, 5, 7, and 8. The longest distance between plants of Genet 1 was 1,577 cm (black circles, Fig. 2).

Observation of underground parts

The excavated parts included 20–30 ramets of

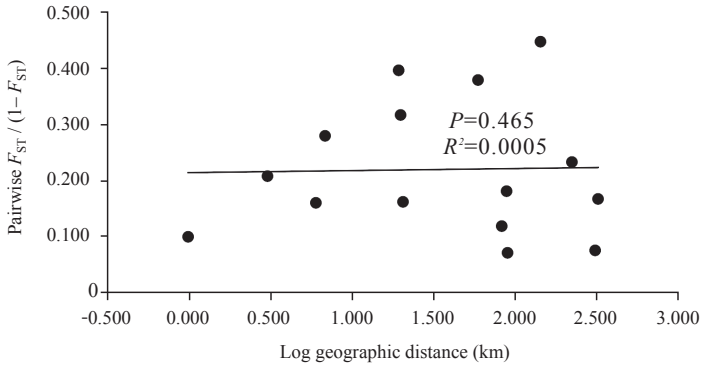

FIG. 3. Relationship between log geographic distance and genetic distance between all pairs of populations of *Veronicastrum sibiricum* var. *zuccarinii*.

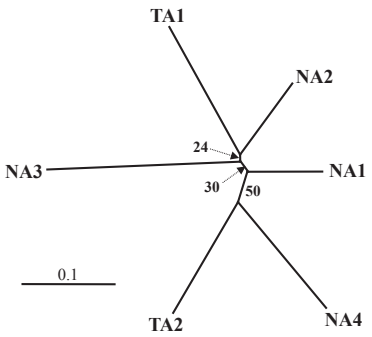

FIG. 4. Neighbor joining (NJ) tree based on genetic distance (D_A) among populations of *Veronicastrum sibiricum* var. *zuccarinii*. Numbers along branches are bootstrap values (%) based on 1,000 replicates. For abbreviations of population names, see Table 1.

Veronicastrum sibiricum var. *zuccarinii* (Figs. 7A, B). Rhizomes with horizontal spread were observed in the underground parts of these plants (Fig. 7C). New shoots for the next year were observed at the base of terrestrial stems (Fig. 7D).

Discussion

Moderate genetic diversity in Veronicastrum sibiricum *var.* zuccarinii

To evaluate whether the genetic diversity of *Veronicastrum sibiricum* var. *zuccarinii* has been lost, we compared the mean number of alleles per locus (N_A), allelic richness (*AR*), and expected heterozygosity (H_E) obtained from microsatellite analyses of additional species of endangered plants in the semi-natural grasslands of the Aso region. For *Echinops setifer* Iljin (Asteraceae), designated as vulnerable (VU) in Japan (Ministry of the Environment of Japan 2015), the values of N_A = 2.1, *AR* = 1.9, H_E = 0.33. Those values were determined using eight microsatellite loci from 10 populations in the Aso region (Kaneko *et al*. 2009). In *Polemonium kiushianum* Kitam. (Polemoniaceae), also endangered (CR) in Japan, the values of N_A = 3.3, *AR* = 2.68, H_E = 0.40. Those values were determined using 10 microsatellite loci from seven populations in the Aso region (Yokogawa *et al*. 2013). In *Primula sieboldii* E. Morren (Primulaceae), designated near threatened (NT) in Japan, the parameters of genetic diversity of the Aso population were N_A= 9.1 and H_E = 0.786, based on eight microsatellite loci (Honjo *et al*. 2009). In the vulnerable (VU) species *Silene kiusiana* (Makino) H. Ohashi & H. Nakai (Caryophyllaceae), five microsatellite loci from seven populations in the Aso region revealed the following values: N_A= 12.0, *AR* = 10.4, and H_E = 0.791 (Yamasaki *et al*. 2013). Moreover, Nybom (2004), using microsatellite marker data, reported mean H_E values in long-lived perennial, narrowly distributed, and outcrossing plants to be 0.68, 0.56, and 0.65, respectively. In the present study of *V. sibiricum* var. *zuccarinii*, we determined the values of N_A = 5.6, *AR* = 4.7, and H_E = 0.596 (Table 3), indicating a moderate degree of genetic variation compared to other semi-natural grassland species, although the populations of var. *zuccarinii* are currently decreasing in size (Environmental Agency of Japan 2000).

Mode of reproduction is known to influence genetic diversity in a species (Hamrick & Godt 1996, Nybom 2004, Duminil *et al*. 2007). For example, Nybom (2004) suggested that the analyses based on microsatellite maker show that long-lived and outcrossing taxa retain most of their genetic variability within populations. *Veronicastrum sibiricum* var. *zuccarinii* is considered to be

capable of outcrossing via insect pollination. From July through August, *V. sibiricum* var. *zuccarinii* forms spike-form inflorescences with numerous, crowded flowers (Kitamura *et al.* 1957, Yamazaki 1993). Although a detailed study of the breeding system of this species has not been performed, a related species, *V. japonicum* (Nakai) T.Yamaz., has hermaphroditic and protandrous flowers that are pollinated by bumblebees, flower flies, and butterflies (Tanaka 1997, 2009). Similar pollinators have also been reported in the related North American species, *V. virginicum* (L.) Farw (Belt 2011, Levin & Kerster 1969, Vaudo *et al.* 2014). Preliminary observations revealed that both bumblebees and flower flies visited flowers among the populations of *V. sibiricum* var. *zuccarinii*. Therefore, var. *zuccarinii* is considered to be outbreeding, although it also reproduces vegetatively (for a discussion of vegetative reproduction, see below). The breeding systems of var. *zuccarinii* may therefore contribute to maintaining moderate genetic diversity within populations.

Another possible explanation for the moderate genetic diversity observed in *Veronicastrum sibiricum* var. *zuccarinii* involves demographic processes (population size changes) in the Aso region. This semi-natural grassland area has decreased from approximately 70,000 ha in 1900 to approximately 36,000 ha around 1990 (National Parks Association of Japan 1995, Shoji 2006). This loss in area has accelerated during the past two decades; the grassland area had been reduced to 23,000 ha in 2010 (Takahashi 2010). We therefore surmise a corresponding decrease in the size of populations of *V. sibiricum* var. *zuccarinii*. In small, fragmented populations, random genetic drift will reduce genetic diversity (Frankham *et al.* 2002). In fact, the evidence of genetic drift was obtained in the Bayesian clustering analysis (Fig. 6), and recent bottlenecks were detected in the NA1 population (Table 1). However, several generations are required before genetic drift has a significant impact, especially in long-lived perennial herbaceous plants (Ohara *et al.* 2006, Tomimatsu & Ohara 2003). In our observations of the underground parts of *V. sibiricum* var. *zuccarinii*, we found evidence of vegetative reproduction by rhizomes (Figs. 2 & 7), suggesting that the individuals of *V. sibiricum* may persist for relatively long periods. Furthermore, such clonal reproduction may decrease the rate at which alleles are lost, enhance heterozygosity, and thereby in-

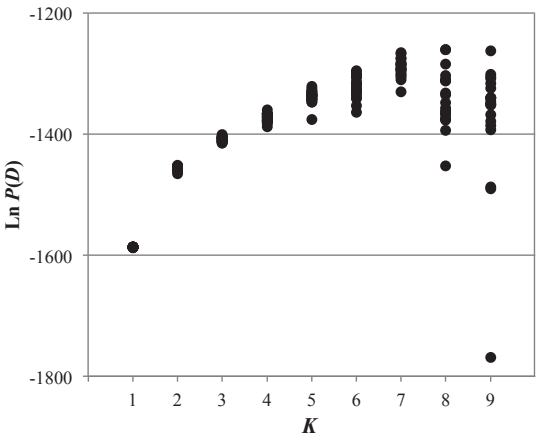

FIG. 5. Distribution of values of log probability of data (Ln P(*D*)) for each cluster (*K*) ranging from 1 to 9 calculated by Bayesian clustering.

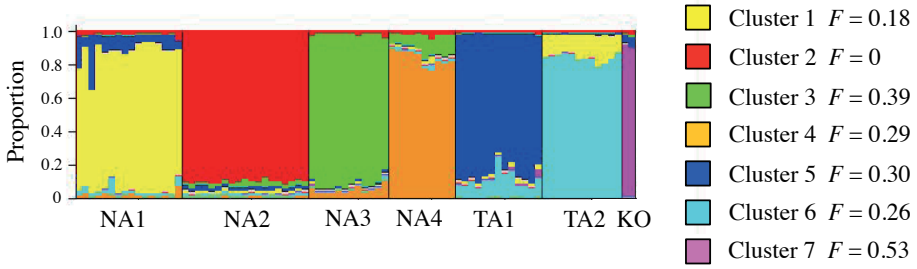

FIG. 6. Proportion of membership of clusters in 234 individuals from seven populations of *Veronicastrum sibiricum* var. *zuccarinii* estimated by Bayesian clustering.

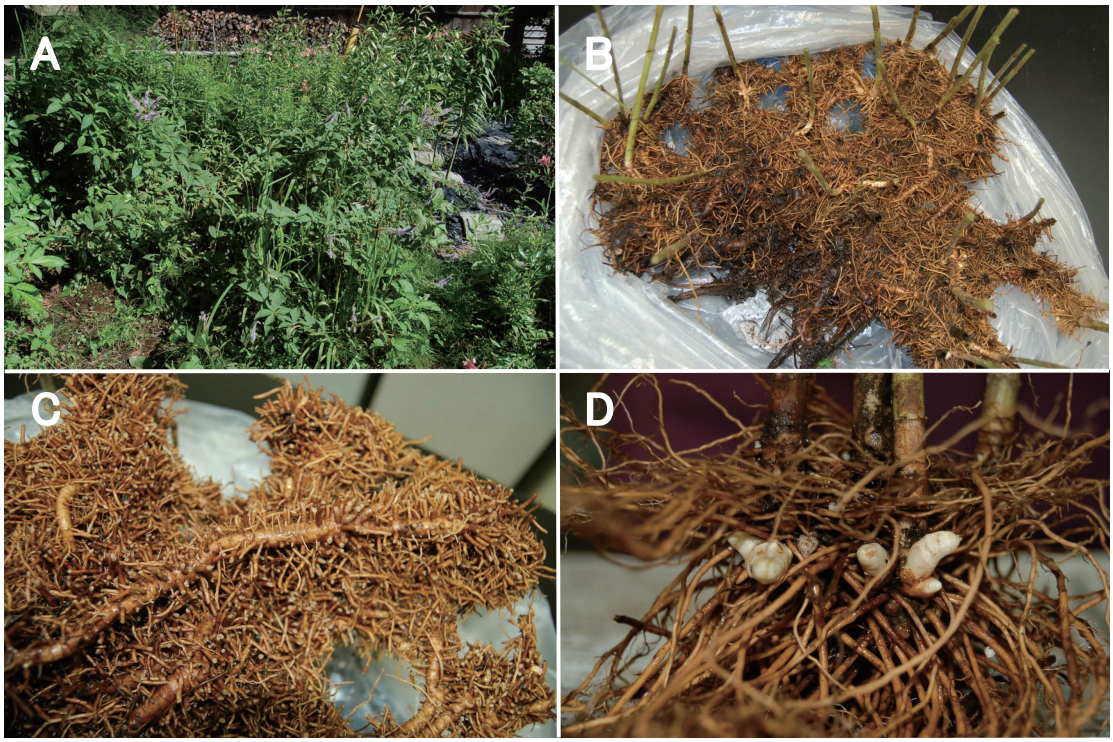

FIG. 7. Horizontally spreading rhizomes were observed below ground in *Veronicastrum sibiricum* var. *zuccarinii*. A: Habitat of observed individuals; B: Upside of stock; C: Closeup of rhizomes; D: New shoots for following year.

crease the effective population size (Balloux *et al*. 2003, Bengtsson 2003). Hence, despite severe habitat reduction, the genetic diversity of *V. sibiricum* var. *zuccarinii* may continue to be maintained.

Genetic differentiation and habitat fragmentation

The values of pairwise F_{ST} (indicating degrees of genetic differentiation among populations) were significantly greater than zero between all population pairs (mean F_{ST} = 0.17, Table 4). In addition, Bayesian clustering, according to Pritchard *et al*. (2000), showed that the clusters of highest proportion of membership (Clusters 1–7) were different in all populations (Fig. 6). Furthermore, both private (*Pr*) and rare alleles (*RA*) were detected in each population (Table 3). Therefore, we consider genetic differentiation to have occurred among populations of *Veronicastrum sibiricum* var. *zuccarinii*.

Over the past several decades, as mentioned above, the habitat of *Veronicastrum sibiricum* var. *zuccarinii* (and other grassland plants) has decreased due to the decline in semi-natural grassland areas in the Aso region (National Parks Association of Japan 1995). In plants that cannot achieve long-distance gene flow, it is known that greater distances between populations because of habitat fragmentation decrease the likelihood of gene flow (Primack & Miao 1992, van Dorp *et al*. 1997, Cain *et al*. 2000, Soons *et al*. 2005). Furthermore, insect-pollinated species with gravity-based seed dispersal are believed to have decreased gene flow between populations when compared with wind-pollinated plants and those with animal- or wind-dispersed seeds (Loveless & Hamrick 1984). As mentioned above, the species of *Veronicastrum* are presumed to be pollinated by insects (bumblebees, flower flies, and butterflies) (Tanaka 1997, 2009, Belt 2011, Vaudo *et al*. 2014). Furthermore, *V. sibiricum* forms capsules with many small (approximately 0.6 mm long), hemispherical seeds (Yamazaki 1993). We

surmise that the seeds are mainly dispersed by gravity because the seeds and capsules lack wind-dispersal appendages. *Veronicastrum sibiricum* var. *zuccarinii* is most likely pollinated by insects and produces gravity-dispersed seeds, making it more susceptible to the effects of habitat fragmentation.

Although we inferred genetic differentiation among populations of *Veronicastrum sibiricum* var. *zuccarinii*, the Mantel test did not reveal a pattern of isolation by distance (IBD) (Fig. 3). In the case of inter-population differentiation without IBD, it is estimated that the effects of genetic drift in each population are greater than those of gene flow (Hutchison and Templeton 1999). In fact, the Bayesian structure analysis (Fig. 6), indicating the degree of genetic drift for each cluster (Falush *et al*. 2003), showed relatively high *F*-values, except for Cluster 2 (0.18−0.53), and recent bottlenecks were also detected in the NA1 population (Table 1). Therefore, it is considered that populations of *V. sibiricum* var. *zuccarinii* are greatly affected by genetic drift because of habitat fragmentation and differences in genetic composition among the population are increasing.

Conservation perspective

We investigated the genetic diversity of *Veronicastrum sibiricum* var. *zuccarinii* from seven populations, which constitute nearly all the known populations of this variety in the Aso region of Japan. Our study revealed that each population has maintained moderate genetic diversity. However, we found evidence of significant genetic differentiation among populations (Table 4). In addition, populations of *V. sibiricum* var. *zuccarinii* in the Aso region are evidently small (estimated roughly as 100–500 ramets/population) (Table 1). Small, fragmented populations are subject to extinction or substantial reductions in population size due to stochastic events (Lande 1993, Melbourne & Hastings 2008), which may lead to genetic erosion depending on the degree to which the effective population size is reduced. Furthermore, in most populations, the number of genets is smaller than the number of ramets (Table 3) and we found individuals in the TA2 population to be actively capable of vegetative reproduction by rhizome (Figs. 2 & 7). By enhancing the variance in genet size and reducing the opportunities for recombination, such clonal reproduction may also reduce the effective population size and consequently enhance the magnitude of genetic drift (Orive 1993, Campbell & Husband 2005). Therefore, we recommend that continuous monitoring be carried out on the population dynamics of *V. sibiricum* var. *zuccarinii*.

In the present analysis, the NA2 and NA1 populations showed higher genetic diversity (N_A, *AR*, *RA*, *Pr*, and H_E) than the other populations (Table 3). Furthermore, the degree of genetic drift in the population was estimated to be relatively small (*F*-values = 0 and 0.18) in the Bayesian structure analysis (Fig. 6). Although we do not know of any specific factors with respect to the genetic status of the populations, both populations have been protected as a semi-natural grassland for many years. The grasslands have been managed by burning and mowing by the Aso Green Stock Foundation, an organization devoted to grassland regeneration (Yamauchi & Takahashi 2002). Accordingly, in these populations, suitable genetic traits may be maintained longer than in other populations. From the viewpoint of conservation genetics, the NA2 and NA1 populations are very important and should be preferentially conserved, although we should conserve as many as possible of the populations, because our analyses showed *V. sibiricum* var. *zuccarinii* exhibits significant genetic differentiation among populations (Tables 3 & 4, Fig. 6).

We estimated the genetic diversity of *Veronicastrum sibiricum* var. *zuccarinii* based on the five microsatellite markers used in the present study. In general, 10 or more markers are frequently used to evaluate the genetic diversity and structure of a population. It is known that estimates of average heterozygosity and genetic distance are affected by the number of loci analyzed (Gorman & Renzi 1979, Nei 1978, Nei & Roychoudhury 1974, Nybom 2004). Therefore, with respect to these parameters, the results should be re-evaluated by increasing the number of markers

that are analyzed.

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